The Temperate Marine Phage ΦHAP-1 of Halomonas aquamarina Possesses a Linear Plasmid-Like Prophage Genome

Jennifer M. Mobberley,1 R. Nathan Authement,2 Anca M. Segall,2 and John H. Paul1*

College of Marine Science, University of South Florida, 140 7th Avenue South, Saint Petersburg, Florida 33701,1 and Department of Biology, San Diego State University, San Diego, California 92182-46142

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A myovirus-like temperate phage, ΦHAP-1, was induced with mitomycin C from a Halomonas aquamarina strain isolated from surface waters in the Gulf of Mexico. The induced cultures produced significantly more virus-like particles (VLPs) \((3.73 \times 10^{10}\) VLP ml\(^{-1}\)) than control cultures \((3.83 \times 10^{7}\) VLP ml\(^{-1}\)) when observed with epifluorescence microscopy. The induced phage was sequenced by using linker-amplified shotgun libraries and contained a genome 39,245 nucleotides in length with a G+C content of 59%. The ΦHAP-1 genome contained 46 putative open reading frames (ORFs), with 76% sharing significant similarity \((E\) value of \(<10^{-7}\)) at the protein level with other sequences in GenBank. Putative functional gene assignments included small and large terminase subunits, capsid and tail genes, an N6-DNA adenine methyltransferase, and lysogeny-related genes. Although no integrase was found, the ΦHAP-1 genome contained ORFs similar to protelomerase and parA genes found in linear plasmid-like phages with telomeric ends. Southern probing and PCR analysis of host genomic, plasmid, and ΦHAP-1 DNA indicated a lack of integration of the prophage with the host chromosome and a difference in genome arrangement between the prophage and virion forms. The linear plasmid prophage form of ΦHAP-1 begins with the protelomerase gene, presumably due to the activity of the protelomerase, while the induced phage particle has a circularly permuted genome that begins with the terminase genes. The ΦHAP-1 genome shares synteny and gene similarity with coliphage N15 and vibriophages VP882 and VHML, suggesting an evolutionary heritage from an N15-like linear plasmid prophage ancestor.

The marine environment is one of the largest reservoirs of viruses, where concentrations range from \(10^7\) virus-like particles (VLPs) per liter to \(10^{11}\) VLPs per cubic centimeter of sediment (5, 65). Viruses are believed to contribute significantly to the marine microbial loop and nutrient cycling in the oceans, and may also serve as agents of gene transfer in the marine environment (19, 43). They may also contribute to the environmental adaptation of their host, as in the case of photosynthetic genes on phages infecting marine cyanobacteria, as well as constrain host diversity (37, 62).

Temperate phages can exist either in a lytic or lysogenic state. In the lysogenic state, the prophage is replicated along with the host genome. Jiang and Paul (28) found that more than 40% of marine bacterial isolates screened contained inducible phages (28). Polysyngeny may also be abundant in the marine environment. For example, the genome of Silicibacter sp. strain TM1040 was found to contain five prophage-like elements, three of which were inducible temperate phages (15). Studies of natural marine populations have indicated that environmental cues, such as host density and temperature, may influence the incidence of lysogeny (36, 64, 66). Although temperate phages are abundant in bacterial isolates and natural environments, little is known about the molecular control of lysogeny in marine bacteria. Sequencing and experimental characterization of temperate marine phage genomes may offer insights into novel lysogenic interactions that occur in the ocean.

Most temperate bacteriophages integrate into the host chromosome during lysogeny. However, some phages, such as Escherichia coli phage P1 and phage cp32 from Borrelia burgdorferi, exist as low-copy-number plasmids (18, 27). E. coli phage N15, Klebsiella oxytoca phage ΦKO2, and Yersinia enterocolitica phage PY54 are a group of closely related phages that exist as linear plasmid-like prophages, with covalently closed hairpin ends (telomeres) due to the activity of a phage-encoded protein, protelomerase (12, 24, 54). During lysogeny, the protelomerase cuts the prophage DNA at an inverted repeat generally located near the protelomerase gene itself. The protelomerase protein resolves the ends of the prophage genome into telomeres. The resulting plasmid prophage gene order is 50% circularly permuted with respect to the virion DNA, such that the terminase genes are found toward the middle of the prophage conformation (24, 54). In addition to a protelomerase, the genomes of these linear plasmid-like phages contain similar lysogeny modules and replication genes, as well as plasmid-partitioning genes, to ensure that daughter cells receive a copy of the phage genome (12, 25, 55). The presence of protelomerase genes in the genomes of the Vibrio harveyi temperate phage VHML and the uncharacterized Vibrio parahaemolyticus phage VP882 indicates that linear plasmid-like prophages may be common among cultivated marine lysogens (39).

Halogomonas aquamarina, formerly known as Deleya aquamarina, is a gram-negative halophilic gammaproteobacterium that has been isolated from a variety of marine and hypersaline environments, including the pelagic ocean, deep-sea hydrothermal vents, the brine-seawater interface of deep-sea brine.
pools, and coastal surface waters (31, 41, 58). The phage-host interactions of two temperate myoviruses infecting *Halomonas* species from the Great Salt Plains in Oklahoma and two temperate siphoviruses from *Halomonas halophila*, isolated from hypersaline soil, have been characterized, but these phages have not been sequenced (11, 60).

To increase our knowledge of marine prophage genomics, we characterized a temperate phage, *ΦHAP-1*, in an *H. aquamarina* isolate from the Gulf of Mexico with respect to morphological characteristics, nucleotide sequence, and overall phage-host relationship. Phage induction resulted in tailded-phage particles resembling members of the *Myoviridae*. Finally, the genomic properties of the phage were experimentally analyzed, including the presence of telomeric ends and its existence as a linear plasmid.

### MATERIALS AND METHODS

#### Isolation of host and phage particles.

*Halomonas aquamarina* was isolated from samples of the surface waters of the Gulf of Mexico (latitude 26°00′N, longitude 83°35.6′W) collected on 15 July 2001. Vortex flow filtration was used to concentrate the water sample (29). The retentate was heated at 80°C for 10 min and then plated onto artificial seawater nutrient agar plates (ASWJP + PY) (45), a procedure employed to select for spore-forming bacteria. The *H. aquamarina* isolate was identified by partial sequencing of a cloned PCR product obtained by using a bacterial 16S universal primer set (23). The bacterium was maintained in pure culture by monthly plating on ASWJP + PY plates, as well as in 25% glycerol stocks kept at −80°C.

*H. aquamarina* phage particles were isolated by a mitomycin C induction procedure. Fifty milliliters of overnight *H. aquamarina* culture was inoculated into 450 ml ASWJP + PY and grown to log phase (optical density at 600 nm, 0.4). Mitomycin C (1 μg ml−1; Sigma) was added to the culture and incubated for 24 h at 28°C. A bacteria-free viral lysate was obtained by centrifugation at 11,000 × g rpm and disruption of the supernatant through 1-μm, 0.4-μm, and 0.2-μm filters. DNase (DNase I) and RNase (RNQ1) (Promega, Madison, WI) were each added (1 μg ml−1), and the reaction mixtures were incubated at room temperature for 30 min. The phage particles were further concentrated with polyethylene glycol 6000 and purified through discontinuous cesium chloride gradients as described by Sambrook and Russell (57) with the following modifications to maximize particle concentration from a marine phage. After the polyethylene glycol 6000 precipitation, the phage pellets were eluted in 2.0 ml of 0.02-M-filtered 75% artificial seawater (ASWJP) (29). The phage particles were purified by centrifugation at 29,000 rpm for 2 h at 4°C through discontinuous cesium chloride gradients of 1.7, 1.5, 1.5, and 1.35 μl in polycarbonate tubes, and the purified phage band was collected from the 1.5-1.35 μl interface by using a syringe.

Phage particles were isolated from a mitomycin C-induced lysate by using Sybr gold stain (Molecular Probes, Eugene, OR) as described by Chen et al. (14), with a staining time of 12 min. VLPs were enumerated by using blue-light excitation and an Olympus BH-2 epifluorescence microscope. At least 200 phage particles in 10 fields per slide were counted.

#### Time series mitomycin C induction of phage particles.

Phage production in *H. aquamarina* was monitored over a 24-h time series following induction with mitomycin C. Five milliliters of an overnight culture of *H. aquamarina* was induced by mitomycin C, and the phage particles were enumerated by using blue-light excitation and an Olympus BH-2 epifluorescence microscope. At least 200 phage particles in 10 fields per slide were counted.

#### Protein analysis by mass spectroscopy.

Phage proteins were separated on a one-dimensional 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel run at 4 mA for 16.5 h by the Laemmli method (33). Gels were fixed in methanol-acetic acid-water (50:10:40), followed by overnight staining (SDS-PAGE) gel run at 4 mA for 16.5 h by the Laemmli method (33). Gels were fixed in methanol-acetic acid-water (50:10:40), followed by overnight staining (SDS-PAGE) gel run at 4 mA for 16.5 h by the Laemmli method (33). Gels were fixed in methanol-acetic acid-water (50:10:40), followed by overnight staining (SDS-PAGE) gel run at 4 mA for 16.5 h by the Laemmli method (33). Gels were fixed in methanol-acetic acid-water (50:10:40), followed by overnight staining (SDS-PAGE) gel run at 4 mA for 16.5 h by the Laemmli method (33). Gels were fixed in methanol-acetic acid-water (50:10:40), followed by overnight staining (SDS-PAGE) gel run at 4 mA for 16.5 h by the Laemmli method (33). Gels were fixed in methanol-acetic acid-water (50:10:40), followed by overnight staining (SDS-PAGE) gel run at 4 mA for 16.5 h by the Laemmli method (33). Gels were fixed in methanol-acetic acid-water (50:10:40), followed by overnight staining (SDS-PAGE) gel run at 4 mA for 16.5 h by the Laemmli method (33). Gels were fixed in methanol-acetic acid-water (50:10:40), followed by overnight staining (SDS-PAGE) gel run at 4 mA for 16.5 h by the Laemmli method (33). Gels were fixed in methanol-acetic acid-water (50:10:40), followed by overnight staining (SDS-PAGE) gel run at 4 mA for 16.5 h by the Laemmli method (33). Gels were fixed in methanol-acetic acid-water (50:10:40), followed by overnight staining (SDS-PAGE) gel run at 4 mA for 16.5 h by the Laemmli method (33). Gels were fixed in methanol-acetic acid-water (50:10:40), followed by overnight staining (SDS-PAGE) gel run at 4 mA for 16.5 h by the Laemmli method (33). Gels were fixed in methanol-acetic acid-water (50:10:40), followed by overnight staining (SDS-PAGE) gel run at 4 mA for 16.5 h by the Laemmli method (33). Gels were fixed in methanol-acetic acid-water (50:10:40), followed by overnight staining (SDS-PAGE) gel run at 4 mA for 16.5 h by the Laemmli method (33). Gels were fixed in methanol-acetic acid-water (50:10:40), followed by overnight staining (SDS-PAGE) gel run at 4 mA for 16.5 h by the Laemmli method (33). Gels were fixed in methanol-acetic acid-water (50:10:40), followed by overnight staining (SDS-PAGE) gel run at 4 mA for 16.5 h by the Laemmli method (33). Gels were fixed in methanol-acetic acid-water (50:10:40), followed by overnight staining (SDS-PAGE) gel run at 4 mA for 16.5 h by the Laemmli method (33). Gels were fixed in methanol-acetic acid-water (50:10:40), followed by overnight staining (SDS-PAGE) gel run![](http://jvi.asm.org/Downloaded from http://jv.asn.org/ on August 15, 2017 by guest)
XbaI for 3 h at 37°C. PFGE was performed as described in reference 43 with a run time of 22.5 h.

Riboprobe labeling and Southern hybridization. A phage riboprobe was created by cloning a PCR-amplified fragment of ϕHAP-1 ORF 37 into the pCR1 cloning vector by using a TOPO cloning kit per the manufacturer’s instructions (Invitrogen, Carlsbad, CA). The probe was created by in vitro transcription using a Riboprobe combination system SP6/T7 (Promega, Madison, WI) according to the manufacturer’s instructions (48). Probes were labeled with 32P-UTP (GE Healthcare, Piscataway, NJ).

A Southern transfer of the pulsed-field gel to charge nylon membranes was done by using the standard method (57). The DNA was cross-linked to the membrane by using an FB-UVXL-1000 UV cross-linker (Fisher Scientific, Pittsburgh, PA). The Southern transfer blot was hybridized and washed as previously described (26). The probed blot was exposed to Biomax high-sensitivity film (Kodak, Rochester, NY) for 3 days at 4°C.

PCR. The two different genomic arrangements of ϕHAP-1 that are due to the activity of the protelomerase were confirmed by designing specific oligonucleotide PCR primer sets (Operon, Huntsville, AL). The primer set for the virion form (genomic arrangement starting with the putative terminase gene) is as follows: primer 1, 5′-GAGAAGTGGGCTGCTCAGA3′; and primer 2, 5′-ATCCTCTACCGCTTCATCCA3′. The PCR product size for this primer set was 2,441 base pairs. The primer set used for the prophage arrangement (genomic arrangement starting with the protelomerase) is as follows: primer 3, 5′-GCTCTCTTGGTTCTGCTCA3′; and primer 4, 5′-GGGTTGTTGCGGAATGTTGATG3′. The PCR product size for this primer set was 2,681 base pairs. The primer set used for the virion form (genomic arrangement starting with the putative terminase gene) is as follows: primer 1, 5′-GAGAAGTGGGCTGCTCAGA3′; and primer 2, 5′-ATCCTCTACCGCTTCATCCA3′. The PCR product size for this primer set was 2,441 base pairs. The primer set used for the prophage arrangement (genomic arrangement starting with the protelomerase) is as follows: primer 3, 5′-GCTCTCTTGGTTCTGCTCA3′; and primer 4, 5′-GGGTTGTTGCGGAATGTTGATG3′. The PCR product size for this primer set was 2,681 base pairs. The DNA preparations were the same as those used in the Southern blot experiment. In order to account for the differing amount of phage DNA present in each preparation, 14 ng of host chromosomal DNA and 2 ng of plasmid or phage DNA were used in the reaction mixtures. GoTaq green mastermix (Promega, Madison, WI) was used as recommended, and the primer sets were added to a thermal cycler (Bio-Rad, Hercules, CA) with the following PCR program: initial thermal cycler (Bio-Rad, Hercules, CA) with the following PCR program: initial annealing of 2 min at 95°C, 25 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 2 min, and a final extension of 72°C for 10 min. The ampiclons were loaded onto a 1% agarose gel with 0.5 µg ml−1 of ethidium bromide and run at 89 V for 45 min. The gel was imaged by using an Alphalmager 2200 imaging system (Alpha Innotech, San Leandro, CA).

Nucleotide sequence accession numbers. The sequence of ϕHAP-1 has been deposited into GenBank under accession number EU399241. The GenBank accession numbers for the phage genomes used in this study are as follows: N15, NC_001901; ϕK02, NC_005857; PY54, NC_005069; VHML, NC_004456; and VP882, NC_009016 (12, 25, 39, 55).

RESULTS

Characterization of the ϕHAP-1 virion. The results of the H. aquamarina phage (ϕHAP-1) mitomycin C prophage induction experiments are shown in Fig. 1. In the mitomycin C-treated cultures, lytic activity was observed, as shown by the number of VLPs being significantly larger in the supernatant than in the control culture (P = 0.01) (Fig. 1B). Based on our sampling regimen, phage production was greatest at 8 h after the addition of mitomycin C (9 × 1010 VLP ml−1) and decreased slightly at 24 h (3.73 × 1010 VLP ml−1). Bacterial growth also decreased after the addition of mitomycin C (P = 0.01) (Fig. 1A). The mean burst size was calculated to be 38 ± 8 (mean ± standard deviation) phages per bacterium.

Electron micrographs of negatively stained ϕHAP-1 particles showed icosahedral capsids with long, thick tails consistent with the Myoviridae family (Fig. 2A and B). The capsid diameter was 49.81 ± 4.89 nm, while the tail was 254.67 ± 17.13 nm in length and 16.09 ± 2.65 nm in width (n = 14).

Analysis of the ϕHAP-1 genome. The ϕHAP-1 genome was found to be 39,245 nucleotides in length and had a G+C content of 59%. No comparisons between the G+C content in the phage and host DNA could be made as the H. aquamarina genome has not been sequenced. The G+C content in ϕHAP-1 is higher than in other protelomerase-containing phages, including PY54 (44%), ϕK02 (52%), N15 (51%), VHML (50%), and VP882 (56%). No cos site was found, since

FIG. 1. Growth of host (A) and prophage production (B) during the 24-h mitomycin C induction experiment. Asterisks indicate when mitomycin C was added. Mean BDC for each time point was used to chart bacterial growth. Error bars indicate the standard deviations of the results from triplicate slides. VDC, enumeration of viruses by the direct-count method.

FIG. 2. Transmission electron micrographs of ϕHAP-1 particles. Black scale bars represent 50 nm.
there was no difference in the pattern of migration observed in the restriction enzyme-digested phage preparations with or without cooling, except for the phage lambda DNA/HindIII digest which served as the positive control (data not shown).

The PhAP-1 genome contains 46 putative ORFs, based on KODON analysis (Fig. 3). Thirty-five of the ORFs (76%) shared significant similarity (E value of \(10^{-3}\)) at the protein level with other sequences in GenBank. Putative functional assignments and significant similarities to other sequences are listed in Table 1. The ORFs identified were similar to genes from other phages or genes encoding phage-related proteins from annotated bacterial genomes. The top BLASTP hits for 29 of the ORFs were similar to those for genes in *V. parahaemolyticus* phage VP882, 20 of which were also similar at the nucleotide level (E value of \(10^{-3}\)) (Table 1).

**FIG. 3.** Genomic map of PhAP-1. KODON was used to construct the gene map. ORFs were numbered based on the arbitrary start of the genome at the terminase small subunit. Patterns were assigned based on functional assignments of the ORFs as indicated in the key.

**ORFs 1 and 2: large and small terminase.** Terminase genes are responsible for ATP-dependent packaging of concatameric DNA in phage capsids. The small subunit possesses DNA recognition specificity, while the large subunit has catalytic activity (7). The ORF 1 product is a hypothetical protein that is similar to vibriophage VP882 on both the protein (E value, \(2e^{-52}\); 55% identity) and nucleotide level (\(5e^{-87}\); 77% identity). This ORF's product also had weak similarity to phage terminase small-subunit nu1 from bacteriophage *H* (\(2e^{-44}\); 30% identity). The ORF 2 product is similar to the putative terminase large subunit in VP882 on both the protein (\(6e^{-59}\); 29% identity) and nucleotide level (\(2e^{-88}\); 75% identity). The ORF 2 product also shares similarity with the terminase large-subunit proteins from *Vibrio cholerae* AM-19226 (\(2e^{-74}\); 53% identity) and *Wolbachia* phage WO (\(4e^{-27}\); 23% identity).

**ORFs 4 and 5: portal and capsid proteins.** Portal proteins are responsible for forming a ring that enables DNA to pass into the major capsid during assembly and out during infection, and they serve as a junction between the capsid and tail proteins (4). ORF 4 encodes a protein similar to the lambda-like portal proteins found in the putative prophage regions in the genomes of *Neisseria meningitidis* FAM18 (\(4e^{-77}\); 35% identity) and *Silicibacter* sp. strain TM1040 (\(1e^{-39}\); 29% identity) and in the *Wolbachia* phage WOcauB1 (\(2e^{-44}\); 29% identity). This ORF shares significant similarity with those found in the vibriophages VP882 (\(2e^{-132}\); 52% identity) and VHML (\(6e^{-13}\); 23% identity). The expected size of the PhAP-1 portal protein, based on the amino acid composition, is 59.4 kDa, which is smaller than the expressed protein identified by MALDI-TOF-TOF from the 65.6-kDa excised band from the SDS-PAGE gel shown in Fig. 4. ORF 5 shares similarity with VP882 (\(2e^{-179}\); 53% identity) and with genes encoding major capsid proteins found in the genomes of *Neisseria meningitidis* FAM18 (\(2e^{-78}\); 33% identity) and *Wolbachia* phage WO (\(7e^{-17}\); 23% identity). Its product also shares weaker identity with the major capsid proteins from the plasmid-like prophage FKO2 (\(3e^{-7}\); 24% identity). MALDI-TOF-TOF analysis of the structural protein profile of PhAP-1 found protein fragments from the major capsid protein in two locations on the SDS-PAGE gel (Fig. 4). Based on the amino acid composition, the predicted size of ORF 5 is 66.6 kDa, while the bands of the gel correspond to 31.5 kDa (ORF 5A) and 28.4 kDa (ORF 5B).

**ORFs 9, 10, 11, 12, 13, 16, 20, 21, 23, 24, and 25: tail proteins.** The PhAP-1 genome encoded 11 putative tail proteins, including three baseplate assembly proteins, a tail fiber protein, a tail tape measure protein, a tail sheath, and a tail tube protein (Fig. 3; Table 1). Tail sheath and tail tubes are components that make up the contractile tail that is the hallmark of the *Myoviridae* family (10). ORFs encoding tail proteins were similar to tail protein genes from myoviruses, in-
<table>
<thead>
<tr>
<th>ORF no./ orientation</th>
<th>Nucleotide position</th>
<th>Predicted function</th>
<th>Related BLASTP hit(s) (accession no.; E value)</th>
</tr>
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<tr>
<td>1/+</td>
<td>1–576</td>
<td>Terminus, small subunit</td>
<td>Hypothetical protein of vibriophage VP882 (YP_001039813; 2e−55); nul of phage λ (AAA96533; 5e−4)</td>
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<td>602–2458</td>
<td>Phage terminase large subunit</td>
<td>Phage terminase large subunit of Vibrio cholerae AM-19226 (EDN16714; 1e−17); phage terminase large subunit of phage WO (BAAX89640; 4e−27)</td>
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<td>2552–3067</td>
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<td>5/+</td>
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<td>Major capsid</td>
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<td>7211–7690</td>
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<td>7671–8231</td>
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<td>Hypothetical protein of vibriophage VP882 (YP_001039825; 3e−4); hypothetical protein Tcr_0686 of Thiomicrospira crunogena XCL-2 (YP_390956; 2e−6); ORF 29 of Vibrio harveyi phage VHML (NP_758922; 5e−5)</td>
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<td>9/+</td>
<td>8240–8863</td>
<td>Baseplate assembly protein V</td>
<td>Phage baseplate assembly protein V of P. fluorescens PFO-1 (YP_346871; 3e−4); phage baseplate assembly protein V of vibriophage VP882 (YP_001039827; 8e−6); baseplate protein of fCTX (NP_490613; 2e−1); gpV of phage P2 (NP_046773; 3e−1); ORF 30 of Vibrio harveyi phage VHML (NP_758923; 1e−7)</td>
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<td>Hypothetical protein of Escherichia coli APEC O1 (YP_852555; 1e−5)</td>
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<td>20/+</td>
<td>14420–15586</td>
<td>Tail sheath</td>
<td>Phage tail sheath protein of vibriophage VP882 (YP_001039839; 2e−145); phage tail sheath protein FI-like of Pseudomonas fluorescens PFO-1 (YP_346877; 9e−12); ORF 39 of Vibrio harveyi phage VHML (NP_758931; 3e−26); phage tail sheath protein of Silicibacter sp. strain TM1040 (YP_613279; 2e−25); phage tail sheath protein of Thiomicrospira crunogena XCL-2 (YP_390965; 2e−16); gpFI of phage P2 (NP_046778; 2e−11)</td>
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<tr>
<td>21/+</td>
<td>15587–16093</td>
<td>Tail tube</td>
<td>Tail tube protein of vibriophage VP882 (YP_001039840; 4e−37); phage-related contractile tail tube of Xylella fastidiosa 9a5e (NP_290818; 1e−7); phage tail tube protein of Thiomicrospira crunogena XCL-2 (YP_390966; 2e−6); ORF 39 of Vibrio harveyi phage VHML (NP_758932; 5e−6)</td>
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<td>22/+</td>
<td>16131–16805</td>
<td>Hypothetical protein</td>
<td>Putative phage-related protein of vibriophage VP882 (YP_001039841; 1e−3); hypothetical protein of P. fluorescens PFO-1 (YP_346879; 2e−26)</td>
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<td>23/+</td>
<td>16930–19515</td>
<td>Tail tape measure</td>
<td>Phage-related tail tape measure of vibriophage VP882 (YP_001039842; 2e−107); putative tail tape protein of phage IFP2 (NP_543009; 1e−50); gpT of phage P2 (NP_046782; 7e−10); phage tail tape measure protein of Thiomicrospira crunogena XCL-2 (YP_390968; 8e−3); ORF 43 of Vibrio harveyi bacteriophage VHML (NP_758934; 1e−6)</td>
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TABLE 1—Continued

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<tr>
<th>ORF no./ orientation</th>
<th>Nucleotide position</th>
<th>Predicted function</th>
<th>Related BLASTP hit(s) (accession no.; E value)</th>
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<td>Tail protein§</td>
<td>Phage protein U-like of vibriophage VP882 (YP_001039843; 2e−35); phage protein U-like of P. fluorescens PFO-1 (YP_346881; 1e−50); F protein of phage 186 (NP_052274; 9e−7)</td>
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<td>25/+</td>
<td>20107–21135</td>
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<td>Phage protein D-like of vibriophage VP882 (YP_001039846; 3e−102); phage protein D-like of P. fluorescens PFO-1 (YP_346883; 8e−47); ORF 46 of Vibrio harveyi bacteriophage VHML (NP_758937; 6e−26); gpD of phage P2 (NP_046784; 3e−18); phage D-like of Thiomicrospira crunogena XCL-2 (YP_390970; 4e−12)</td>
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<td>21276–22031</td>
<td>DNA adenine methyltransferase§</td>
<td>DNA adenine methyltransferase of vibriophage VP882 (YP_001039848; 5e−109); DNA adenine methyltransferase of ΦE125 (NP_53663; 1e−12); DNA adenine methyltransferase of phage F116 (YP_164275; 2e−22)</td>
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<td>24358–24954</td>
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<td>25561–25860</td>
<td>Hypothetical protein of vibriophage VP882 (YP_001039863; 2e−11)</td>
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| 33/+                 | 25853–26617         | Partitioning protein§ | Nucleotide similarity to VP882 is 70% identity) and including P2, P1, VHML, and ΦCTX. Six of the ΦHAP-1 ORFs (11, 16, 20, 21, 23, 25) were significantly similar to genes of the inducible prophage from Thiomicrospira crunogena XCL-2 (Table 1). Many of the ORF products were similar to proteins encoded in a prophage-like region on the Pseudomonas fluorescens PFO-1 genome. All the putative ΦHAP-1 tail proteins shared significant similarity with proteins from the VP882 phage. The tail sheath protein (encoded by ORF 20) was identified from the structural protein profile of ΦHAP-1; the predicted size of the protein (41.7 kDa) is smaller than the expressed size of the protein (43.6 kDa) (Fig. 4). 

**ORF 26: DNA-methylating protein.** ORF 26, which is immediately downstream of the tail genes, putatively encodes a DNA adenine methyltransferase protein. Methyltransferase genes are believed to methylate phage DNA in order to protect it from host restriction endonucleases (1). This ORF’s product has significant similarity to proteins from other temperate phages infecting gram-negative bacteria, including from phage VP882 (5e−109; 72% identity), and the N6 adenine methyltransferase from the Burkholderia mallei phage ΦE125 (1e−93; 62% identity) and Pseudomonas aeruginosa phage F116 (2e−22; 31% identity). Additionally, ORF 26 shares 73% nucleotide identity across 90% of the gene with VP882 and 68% identity over 92% of the gene with ΦE125.

**ORFs 33, 34, and 36: genome maintenance module.** This group of ORFs is similar in sequence and organization to those
found in other plasmid-like temperate phages. ORF 33 potentially encodes a partitioning protein (ParA) that was similar to gene products from phage VP882 (1e-65; 60% identity), the product of ORF 58 of phage VHML plasmid pRA2 (3e-51; 53% identity). ParA is part of the system that is responsible for plasmid segregation during cell division (21). ORF 34 is similar to protelomerase genes found in linear plasmid-like phages, including N15 (3e-64; 33% identity), ΦKO2 (7e-54; 32% identity), and PY54 (2e-50; 32% identity). Protelomerases process phage DNA at a specific inverted repeat, resulting in linear phages with covalently closed ends (16). This ORF also shared significant similarity with a gene from VP882 (3e-155; 54% identity). ORF 36 encodes a replication protein similar to those in other plasmid-like prophages, such as the repA in PY54 (1e-119; 29% identity), N15 (1e-112; 29% identity), and ΦKO2 (2e-113; 29% identity). RepA is a multifunctional replication protein with primase and helicase activities that is found in plasmid-like phages (63). Additionally, these three ORFs shared significant nucleotide similarity with genes from VP882, with at least 65% identity along 42 to 71% of the gene (Table 1).

**ORFs 37, 38, and 42: lysogeny module.** ΦHAP-1 contains lysogeny-related genes found in known temperate phages. The gene products include a putative phage repressor (encoded by ORF 37) that is upstream from and in reverse orientation to a conserved phage protein (encoded by ORF 38) and the transcription antiterminator Q protein (encoded by ORF 42). Repressors are the regulators of lysogeny by binding to promoter sites that prevent the transcription of lytic genes (51). The antiterminator Q protein is not involved in the lytic-lysogenic decision, but it must accumulate in order to allow the transcription of late lytic genes (32). The ORF 37 product contains a conserved helix-turn-helix DNA binding domain that is similar to a gene product from VP882 (8e-23; 31% identity). It also shares similarity with a prophage repressor protein from *Burkholderia* phage BcepC6B (4e-7; 26% identity) and the cI repressor of phage 434 (6e-7; 21% identity). The ORF 38 product is a conserved phage protein similar to the products of ORF 7 of VHML (1e-29; 37% identity) and ORF 60 of VP882 (5e-29; 38% identity). ORF 38 also shares similarity with *cro* from PY54 (4e-18; 26% identity), which competes with cI for binding sites for the operator to initiate the lytic cycle (51). ORF 42, encoding the putative transcriptional antiterminator Q, is located 1,380 base pairs downstream from ORF 38. The ORF 42 product is similar to proteins from VP882 (3e-20; 43% identity), as well as *q* proteins from PY54 (1e-3; 29% identity) and the enterobacterial phage 82 (3e-7; 31% identity). No integrase or lytic genes were identified in the ΦHAP-1 genome by similarity searches.

**Inverted repeat.** Since N15 contains an inverted repeat site that enables the protelomerase to break the phage genome and rejoin it to make the linear telomeres, we searched the ΦHAP-1 genome for an inverted repeat. A 92-base-pair inverted repeat, palindromic repeat was found between the regions encoding the partitioning protein (encoded by ORF 33) and the protelomerase (encoded by ORF 34) (Fig. 5). The end of the repeat was found to be 127 base pairs upstream from the start codon of the gene encoding the protelomerase protein.

**Genomic organization of plasmid-like, double-stranded linear prophages.** A comparison of the genomic organization of ΦHAP-1 with the genomes of temperate phages that harbor a protelomerase gene (e.g., linear plasmid prophages N15, PY54, and ΦKO2 and vibriophages VHML and VP882) is shown in Fig. 6. There was no nucleotide sequence homology between ΦHAP-1, N15, PY54, ΦKO2, or VHML, but the organization of structural, lysogeny, and replication modules in these phage genomes was strikingly similar. The linear plasmid...
prophages N15, PY54, and KKO2 are longer than ΦHAP-1 and the vibriophages and are the only ones that contain rec-ognizable lytic genes. The nucleotide sequence similarity, with a minimum of 60% identity over at least 80 bases, between ΦHAP-1 and VP882 was seen across the genomes, including in most of the structural, DNA metabolism, and plasmid-like genes, but not in the lysogeny-related genes. VP882 contained genes for a putative transcriptional regulator and exonuclease that are not present in ΦHAP-1.

Integration of ΦHAP-1 in cellular replicons and different phage genomic arrangements. Southern hybridizations of host chromosomal, plasmid, and phage preparations were performed to determine if ΦHAP-1 integrates into the chromosome or exists as an extrachromosomal element. Nucleic acid preparations were restriction digested at unique sites or double digested to confirm assembly. Figure 7 illustrates the different digestion sites for the restriction enzymes based upon two hypothetical structures derived from similarities of phage N15 structure. The undigested DNA preparations hybridized at a molecular weight of 39 kb (Fig. 8, lanes 2, 7, and 12), indicating a lack of integration into the host chromosomal DNA. The BamHI-digested preparations served as a positive control for the presence of phage DNA in the fractions. In the host and plasmid fractions, these hybridized at around the same molecular weight (10 kb), with the fragment in the phage being slightly larger (Fig. 8, lanes 6, 11, and 16). The hybridization patterns of the rest of the host genomic and plasmid digests were identical, while the phage virion digestion pattern was different (Fig. 8, lanes 3 to 5, 8 to 10, and 13 to 15).

The detection of the two different phage genomic arrangements was verified by PCR analysis of the host genomic, plasmid, and cesium chloride-purified phage preparations using the two form-specific primer sets (Fig. 7). The virion primer set (primers 1 and 2) resulted in an amplicon 2,441 base pairs in length, while the prophage primer set (primers 3 and 4) resulted in a fragment 2,681 base pairs in length (Fig. 9). The host genomic (Fig. 9, lane 3) and plasmid (lane 5) amplicons were from the prophage primers, with no amplification with the virion primers. For the phage preparation, there was only amplification from the virion primer set (Fig. 9, lane 6). We interpret the similarity of the host genomic and plasmid preparations as being caused by the presence of plasmid DNA in the host genomic preparation.

DISCUSSION

Based on the TEM micrograph showing thick, contractile tails, ΦHAP-1 can be classified as a myovirus. The diversity and number of ΦHAP-1 ORFs encoding tail proteins, including ORFs with products similar to a tail sheath, tail tube, and several baseplate proteins from P2-like temperate myoviruses that have genomes below 50 kb in size, including coliphage P2, VHML, and ΦCTX, support this finding (35, 38, 39). The major structural components of the contractile tails that are
the hallmark of the Myoviridae family are the tail tube and tail sheath proteins, both of which are found on the PhAP-1 genome. The tail sheath protein, which contracts during infection of the host, was identified by peptide sequencing of a protein band from the PhAP-1 proteome expressed on an SDS-PAGE gel. The actual molecular mass of the tail sheath protein agrees with the predicted molecular mass, indicating little posttranslation modification. The tails of PhAP-1 particles are longer and have less variation in length than those of the other, related myoviruses P2 and VHML (6, 40). The length of the tail tape measure protein, which was identified in PhAP-1 as the product of ORF 23, is believed to be directly proportional to the phage tail length (30). This protein in PhAP-1 was 861 amino acids long, which puts its length between those of P2 and VHML.

The capsid-associated proteins were similar to those from lambda-like siphoviruses, such as WOcauB1, PY54, and PhKO2 (12, 20, 24). The expression and identification of the major capsid and portal proteins on the SDS-PAGE gel confirmed our in silico annotation of these head genes and provided further information on the mature PhAP-1 virion. The major capsid protein is typically the most-abundant protein present in mature virions, and so it is highly expressed on SDS-PAGE gels, as is seen in Fig. 4. Sequencing of the peptide fragments from two different-sized protein bands suggests that the PhAP-1 major capsid protein is cleaved after translation into two smaller proteins. The sum of the two fragments is 59.9 kDa, which is close to the predicted size of 66.6 kDa for the major capsid protein. Proteolytic cleaving of the viral-coat proteins during assembly occurs in several phages, including PY54 and PhKO2 (12, 25). The expressed PhAP-1 portal protein is 6 kDa larger than the molecular mass that is expected based on the amino acid sequence, indicating either abnormal migration in the SDS-PAGE gel or posttranslation modification of the protein. The similarity of PhAP-1 capsid and tail proteins to those from mitomycin C-inducible phages found in the genomes of the deep-sea chemolithoautotroph T. crunogena and the Pfisteria piscicida-associated bacterium Silicibacter sp. strain TM1040, as well as to marine vibriophage VHML, illustrates the conservation of structural genes in marine prophages (15, 59).

PhAP-1 is a double-stranded DNA phage. No cos site was found in the genome, but due to the presence of terminase genes, we propose that PhAP-1 is packed by the headfull packaging mechanism. Packaging of phages via this mechanism begins from the rolling-circle replication intermediate at a sequence usually known as a pac site and proceeds until no more DNA fits into the capsid, at which point the phage DNA is cut and the rest is packaged into the next capsid (7). This results in DNA of different lengths with no specific sequence at the ends of the majority of the packaged phage genomes. These types of tailed phages have circularly permuted genomes; thus, the terminase small subunits are often selected as the arbitrary start points for genomic maps to ease comparison between phages (13, 46). This convention was followed when constructing the PhAP-1 genomic map.

The H. aquamarina host behaved like a lysogen when treated with the prophage-inducing chemical mitomycin C. Mitomycin C is an antibiotic that inhibits DNA synthesis in bacteria by cross-linking cDNA strands, eliciting the SOS response and causing prophage induction (42). The increase in the number of VLPs in the mitomycin C-treated culture in comparison to the lack of change in the number of VLPs in the control treatment was typical of a lysogenic bacterium.

![Fig. 7. Schematic representation of the two conformations of the PhAP-1 genome. The cut sites of restriction enzymes are indicated by vertical lines. The predicted sizes of fragments are noted to the right of each map. The numbered arrows below each map represent the primers used for PCR analysis. Figures are not drawn to scale. Pt, protelomerase; Pa, ParA; Ts, terminase small subunit; 46, ORF 46 hypothetical protein; cl, ORF 36 prophage repressor, which was used as the probe.](http://jvi.asm.org/on August 15, 2017 by guest)
As φHAP-1 is mitomycin C inducible, the presence of several lysogeny-related genes on the φHAP-1 genome is not surprising. The prophage repressor and antirepressor are responsible for determining if the phage will enter the lytic or lysogenic cycle. The location and orientation of these two proteins in the φHAP-1 genome are characteristic of repressors in the model phage system λ (51). Functional studies of coliphage 434 and PY54, which share similarities with the prophage repressor and antirepressor, respectively, of φHAP-1 indicate that these gene products act as repressor and antirepressor in these model systems (25, 47). In φHAP-1, the ORF encoding the transcription antiterminator Q protein, which is part of the lytic switch that activates late gene expression, is located downstream from the repressor protein ORFs. This protein is similar to the antiterminator Q protein from enterobacterial phage 82, whose activity was shown during protein activity assays (22). The lack of identifiable lytic genes in φHAP-1 is not unprecedented. It is possible that some of the unknown φHAP-1 ORFs 27 through 31 could be lysis-associated genes, although functional protein studies would need to be undertaken to determine this. No lysis-associated genes were found in the genomes of the lytic roseophage SIO1 or the pseudo-temperate phage φHSIC, both of which we isolated from ma-

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FIG. 8. Southern gel transfer (right) and PFGE (left) of φHAP-1 DNA fractions. Lanes: 1, 8- to 48-kb standard; 2 to 6, host chromosomal DNA (2, undigested; 3, XbaI and Nar I digested; 4, Nar I digested; 5, XbaI digested; 6, BamHI digested); 7 to 11, H. aquamarina plasmid DNA (7, undigested; 8, XbaI and Nar I digested; 9, Nar I digested; 10, XbaI digested; 11, BamHI digested); 12 to 16, φHAP-1 DNA (12, undigested; 13, XbaI and Nar I digested; 14, Nar I digested; 15, XbaI digested; 16, BamHI digested). Numbers at left are sizes in kilobases.

FIG. 9. Gel electrophoresis of H. aquamarina DNA PCR ampli-cons. Lanes: 1, MidRange Plus DNA ladder, with sizes in base pairs on the left; 2, host chromosomal DNA amplified with primer set 1 and 2; 3, host chromosomal DNA amplified with primer set 3 and 4; 4, H. aquamarina plasmid DNA amplified with primer set 1 and 2; 5, H. aquamarina plasmid DNA amplified with primer set 3 and 4; 6, φHAP-1 DNA amplified with primer set 1 and 2; 7, φHAP-1 DNA amplified with primer set 3 and 4.
rine bacteria (46, 56). These marine phages most likely have lytic genes, but they remain unknown due to the bias of genomic databases toward terrestrial and medical phages.

Although no recognizable lysis genes are present in the genome, the ΦHAP-1 genome contained an ORF encoding an N6-DNA adenine methyltransferase. Many tailed-phage genomes contain methyltransferase genes, whose products are believed to methylate phage DNA to protect it from host restriction endonucleases, providing a selective advantage over unmodified phages (1). Phage methyltransferases may also play a role in host pathogenicity through lysogenic conversion (39). For ΦE125, the function of the N6-DNA adenine methyltransferase was confirmed through protein expression (67). In N15 and ΦKO2, adenine methyltransferases were expressed late during lytic growth and were not detected during lysogeny (12, 55). In temperate phage genomes, methyltransferases are located in the lytic or DNA modification modules (39, 40, 55).

The presence of three genes in ΦHAP-1 that are similar to those in linear plasmid-like phages and bacterial plasmids and the absence of an integrase suggest that ΦHAP-1 exists as a linear plasmid. This was also supported by restriction digestion analysis and PCR of the plasmid and phage genomic preparations. Partitioning proteins, known generically as ParA and ParB, ensure that daughter bacteria will contain copies of bacterial chromosomal and low-copy-number plasmids (21). Both ParA and ParB are necessary for stable inheritance of the N15 prophage (53). Although the ΦHAP-1 parA was similar to those in the two vibriophages and a bacterial plasmid gene, there was no parB homolog. This may indicate that the partitioning mechanism in this phage is different from the ParA-ParB system in coliphage N15. In N15, RepA is a multidomain protein containing conserved primase, helicase, and replication origin sites whose activity is similar to the theta replication mechanism found in some plasmids (54, 63). The replication protein (RepA) of ΦHAP-1 is very similar to those of linear plasmid phages, such as N15. Ravin (54) also found that RepA was active during lytic and lysogenic growth, allowing maintenance of the plasmid state.

Protelomerase genes have been found in a small number of phages, including the temperate linear plasmid siphoviruses N15, PY54, and ΦKO2 and the vibriophages VHML and VP882 (12, 25, 39, 55). In N15, this gene was found to be necessary for the replication of the prophage form, but not the virion form (54). The proteolomerase is responsible for breaking and resolving the single-stranded DNA ends into hairpin ends, allowing them to exist as a linear plasmid (16, 24). Although the exact mechanism is unknown, the results of studies with proteolomerase from both N15 and ΦKO2 suggest that two proteolomerase molecules form a dimer on a 10-base-pair core palindromic inverted repeat. Each molecule is responsible for cutting and rejoining the strands to form hairpin ends (17, 26). The inverted repeats in these linear phages are found about 150 base pairs upstream from the proteolomerase gene and are between 42 and 56 base pairs in length. The 92-base-pair palindromic inverted repeat identified in ΦHAP-1 was much longer than those in the linear plasmid prophages. Like the one in PY54, it was also palindromic for its whole length, unlike those in N15 and ΦKO2, which are interrupted by a 2-base-pair insertion. These differences could result in differences in the binding affinities of the proteolomerases, although further experiments are needed.

The results of Southern hybridization and PCR experiments indicate that ΦHAP-1 does not integrate into the host chromosome but exists as a linear episome. An integrated phage would have hybridized with the phage probe at a much-higher molecular weight in the undigested host DNA than in the phage and plasmid DNA fractions. This was not the case in the results of our Southern hybridization experiment, as seen by the presence of the 39-kb ΦHAP-1 genome-sized fragment in all of the DNA preparations in Fig. 8. The linear nature of the ΦHAP-1 phage genome was seen in the phage DNA fraction digested with the unique cutter restriction enzyme, Nar I. If ΦHAP-1 was a circular plasmid, the single restriction cut would linearize the molecule, causing it to migrate as an apparently higher-molecular-weight molecule. In the case of our Southern hybridization results, the target fragment size was the same as predicted by the linear virion restriction maps (Fig. 8).

As a prophage, ΦHAP-1 exists as a linear plasmid. In this arrangement, the genome starts with the proteolomerase gene and ends with the gene for the partitioning-protein ParA, most probably due to the activity of the proteolomerase. The prophage form was dominant in the host genomic and plasmid DNA fractions, as these cultures were not induced with mitomycin C treatment. This is apparent in the digestion patterns of the host genomic and plasmid DNA, where the hybridized fragments were the same as those predicted by the prophage restriction maps (Fig. 8). Additionally, only the prophage primer set (primers 3 and 4) amplified the host and plasmid DNA. The differences between the host genomic and plasmid fractions in the hybridization strength and amounts of PCR amplicon were indicative of the low concentration of phage DNA present in the whole-host DNA fractions relative to the amount in the plasmid preparations.

In the virion, the ΦHAP-1 genome is a linear, circularly permuted molecule; as such, the terminase genes were used as the start point. The phage DNA used in the hybridization and PCR experiments was isolated from a large-scale mitomycin C induction experiment, and so the virion form dominated. The differences between the results of the restriction digests and Southern hybridization of the phage DNA in comparison to those of the host genomic and plasmid DNA support this arrangement. In the PCR experiment, only the virion primer set (primers 1 and 2) amplified phage DNA. These findings are similar to what was seen in the results of the restriction digest experiments for other linear plasmid phages, including N15, PY54, and ΦKO2 (12, 25, 55).

ΦHAP-1 shares genetic characteristics with the other phages that contain a proteolomerase gene, the N15-like linear plasmid phages N15, PY54, and ΦKO2 and two marine vibriophages, VP882 and VHML. The overall genomic organization of the functional modules was similar across all six phages, with packaging, structural, and phage metabolism genes present. Conservation of functional gene groups, even if the genes themselves are not similar, is common among temperate tailed phages, including those from the marine environment (9, 44). This conservation supports Botstein’s modular theory of phage evolution, where evolution occurs through the exchange of groups of functional genes (modules) between phages, and these modules select for optimal biological activity in a partic-
ular environment (8). In ΦHAP-1, genes within the modules share similarity with those of a variety of siphoviruses and myoviruses. This mosaicism supports what is seen in other marine phages, such as the prophage of *Thiomicrospira crunogena* XCL-2 and vibriophages K139, VP16T, and VP16C (44, 59).

Phages that contain a protelomerase, with the exception of VP882 and VHML, which have not been characterized on that level, exist as stable plasmid prophages. It is possible that a plasmid-like prophage conformation could be advantageous to the phage, not requiring the constraints that a chromosomally level, exist as stable plasmid prophages. It is possible that a "Phage's genome structure."

The genome of the temperate *H. aquamarina* phage ΦHAP-1 is an important addition to the growing number of marine prophages that have been characterized to date. To our knowledge, ΦHAP-1 is the first marine temperate phage characterized to have behaved like a telomeric, linear plasmid-like phage. Combining traditional host-phage characterization with molecular studies in this system has provided insight into the phage's genome structure.

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2. Reference deleted.